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13. ABSTRACT (Maximum 200 Words)

uPAR (urokinase-type plasminogen activator receptor) is a key player in metastasis of breast cancer cells. We suggest that uPAR, because it is a GPI-anchored protein, must be present in discrete "rafts" in the cell surface to function. Our proposal has two parts. First, we will set up systems in our lab for studying signaling through uPAR in cultured human breast cancer cells. Second, we will disrupt rafts, and determine whether signal transduction is affected. Our most important advance this year has been in developing new tools for raft disruption. These include sterol analogs such as androstanol and coprostanol. Replacing cholesterol with these analogs allows us to disrupt rafts without depleting total cellular sterol, allowing raft disruption without the other pleiotropic effects that accompany bulk sterol removal. This will be an important tool in later experiments to examine the effect of raft disruption on uPAR-mediated signaling and cell motility. We anticipate in the next year, we will develop improved methods for detecting uPAR in rafts in cells. We will then determine how the localization of uPAR in rafts governs its deadly activity in metastasis.

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Table of Contents

INTRODUCTION

Signaling through lipid rafts (membrane microdomains enriched in cholesterol and sphingolipids) plays a key role in normal cell homeostasis and in aberrant proliferations and metastasis of breast cancer cells. This occurs as a high degree of partitioning of certain signaling proteins into rafts favors their interaction and enhances signaling. Thus, understanding how these proteins concentrate in lipid rafts, and how raft localization aids signaling, will aid in blunting the transformed phenotype. GPI-anchored proteins, which are anchored in membranes through covalent linkage to glycosyl phosphatidylinositol, are highly enriched in rafts. One GPI-anchored protein, uPAR (urokinase-type plasminogen activator receptor), is a key player in metastasis of breast cancer cells. We proposed that uPAR must be present in rafts to function. The rationale for this hypothesis comes from previous work, which has shown that a number of GPI-anchored proteins can participate in signal transduction cascades. Importantly, in several cases (especially cells of the immune system), these proteins must be present in rafts in order to send signals. Because uPAR is also lipid-anchored, we hypothesize that it must also be in rafts to send signals. Our proposal had two parts. First, we endeavored to examine the signaling and raft localization behavior of uPAR and other GPI-anchored proteins in cultured human breast cancer cells. Our second goal was to disrupt rafts, and determine whether signal transduction was affected. We proposed three different means of disrupting rafts, in each case altering the lipid composition of the membrane. All three methods were based on knowledge of how lipids are organized in rafts. We found that lipids in rafts are packed tightly together. Agents that counteract this tight packing were expected to disrupt rafts, and thus to inhibit uPAR signaling.

BODY

Task 1. To establish systems for uPAR signaling in breast cancer cells in our lab, and to determine whether uPAR functions observed in other cells are also seen in breast cancer cells. We continued to be plagued by difficulties in detecting and examining uPAR with available commercial reagents. Over the course of the award period, we attempted several different strategies of overcoming this stumbling block. We started by trying to detect uPAR in MCF7 breast cancer cells by standard immunofluorescence microscopy, using commercially available antibodies (American Diagnostica, Inc). We detected only a very dim, background-like staining. Increasing the concentration of primary or secondary antibodies did not improve specific staining, although non-specific background staining (of similar intensity with or without primary antibodies) was observed as the concentration of secondary antibodies was increased. In an attempt to improve weak staining, we tried secondary antibodies linked to a variety of fluorophores. These included fluorophores of the Alexa series from Molecular Probes, often considered to be more intense than the conventional fluorescein, rhodamine, or Texas red stains. We also attempted to enhance staining using a "sandwich" technique, adding an additional layer of antibodies to amplify the signal. None of these approaches were successful.

It seemed possible that MCF7 cells might contain relatively low levels of uPAR. If so, and if the antibody was sub-optimal, better results might be obtained with different cells. For this reason, we also examined 3 other breast cancer cell lines; MCA-MB-231 (reported to express high levels of uPAR), MDA-MB-435, and SKBR3. We were unable to obtain unambiguous results on any of these lines. Finally, hoping to obtain positive results even if it were not in breast cancer cells, we examined primary HUVEC (human umbilical vein endothelial cells), obtained in collaboration with Dr. Martha Furie (University at Stony Brook, Stony Brook NY), expected to be a rich source of

uPAR. Although we detected a dim cell-surface stain in these cells, it was not sufficient for further analysis. As we routinely perform immunofluorescence microscopy, detecting other proteins in other cell types, in the laboratory, our methodology appears to be adequate for this technique.

We also attempted to detect uPAR by Western blotting, using the same antibodies. All of the cell and tissue sources described above were examined. We were unable to unambiguously detect the ca. 50 kDa uPAR protein, above the considerable background, from any of these. Various conditions of antibody concentration, blocking, and sample pre-treatment were without effect. Similar results were obtained using nitrocellulose or nylon membranes.

Examination of additional GPI-anchored proteins. To work around this difficulty, we took parallel approaches to the problem, with much greater success. The first was to examine other GPI-anchored proteins. Because different GPI-anchored proteins associate with rafts in a similar manner, we expect that initial studies on these proteins will be directly applicable to uPAR. Specifically, we have examined the GPI-anchored protein placental alkaline phosphatase (PLAP) as a model. We demonstrated that we could detect transfected PLAP cleanly on the surface of transfected MCF7 human breast cancer cells (Figure 1). Further studies with PLAP, as a model for uPAR, are described below and in the next section (Task 2).

Raft disruption by cholesterol depletion stimulates the MAP kinase pathway. We also initiated studies of the role of raft integrity in signal transduction. As discussed below (Task 2), we made significant progress in developing useful assays for raft disruption. Previous work by us and others has shown that raft integrity and function are completely dependent on membrane cholesterol (Ostermeyer *et al.*, 1999; Sheets *et al.*, 1999). Thus, we disrupted rafts by depleting cholesterol. Treatment of cells with methyl beta cyclodextrin (MBCD) is the most widely-used method for disrupting rafts in cells. Cyclodextrins are a class of compounds that form hydrophobic pockets in otherwise hydrophilic molecules. Some cyclodextrins bind specifically to cholesterol in this hydrophobic pocket (Kilsdonk *et al.*, 1995). Thus, high concentrations of MBCD added to cells extract cellular cholesterol into the binding pocket, converting it to a soluble, non-cell-associated form that can easily be washed away. Adding high concentrations of cyclodextrins to cells rapidly and efficiently extracts a high fraction of the total cellular cholesterol (Kilsdonk *et al.*, 1995). MBCD efficiently removes up to 70-80% of total cellular cholesterol in less than an hour, and has turned out to be a very useful tool for disrupting rafts (Ilangumaran and Hoessli, 1998). We used this method to deplete cholesterol from cells grown in tissue culture dishes.

Fig. 1. Immunofluorescence localization of PLAP. MCF-7 cells transfected with PLAP were incubated with anti-PLAP and then with fluoresceinated goat anti-rabbit IgG, and visualized by fluorescence.

Importantly, MBCD treatment is readily reversible. Cholesterol can be pre-loaded into MBCD, and MBCD-cholesterol complexes added to cholesterol-depleted cells can efficiently deliver cholesterol back to the cells, to levels as high or higher than are normally present there. This approach has shown that MBCD treatment is not directly toxic to cells. Thus, cholesterol can be removed for at least several hours, and then restored by addition of MBCD-cholesterol complexes, with no long-term toxicity to the cells. Additional information on our characterization of the effects of MBCD treatment on rafts in breast cancer cells is presented in Task 2.

Here, we report the effect of this disruption on signal transduction pathways culminating in activation of MAP kinase. A useful model for migration of breast cancer cells during metastasis is to examine the stimulated motility of cultured breast cancer cells in vitro. Previous work has shown

that stimulated motility of breast cancer cells involves signaling cascades that culminate in activation of MAP kinase (Nguyen *et al.*, 1998). Our hypothesis is that this signaling pathway involves the function of, and is regulated by, lipid rafts.

To examine the effect of cholesterol depletion and raft disruption on signaling though the MAP kinase pathway, we took advantage of the availability of antibodies that specifically recognized activated MAP kinase. MAP kinase becomes phosphorylated when it is activated. Some antibodies recognize only the phosphorylated form. Other control antibodies are also available that recognize MAP kinase in both active and inactive forms. In the experiment shown in Figure 2, we either treated cells with methyl-beta cyclodextrin (MBCD), (marked +) or with vehicle alone (marked -). We then lysed the cells under conditions in which the phosphorylation status of proteins would be maintained. The cell lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS PAGE). Proteins were then transferred to nitrocellulose for Western blotting. The blot was first probed with the activation-specific antibody. As shown in Figure 2, left panel, the activated form of the protein is only seen after MBCD treatment. As a control, to ensure that equal amounts of protein were actually present, the blot was stripped and reprobed with antibodies that recognize the protein regardless of activation state. As shown in Figure 1, right panel, protein levels were very similar in both cases. We conclude, then, that raft disruption greatly stimulates signaling through the MAP kinase pathway.

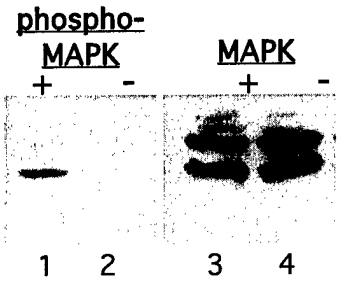


Figure 2. MBCD activates MAP kinase. Cells were (+) or were not (-) treated with MBCD before lysing and analyzing by Western blotting, probing for activated (lanes 1 and 2) or (3 and 4) total MAP kinase.

Yes is present and active in membrane rafts, and tyrosine phosphorylates a number of cellular proteins. Non-receptor tyrosine kinases of the Src family have been implicated in signal transduction in membrane rafts in a number of different cell types (Brown and London, 1998a). However, it has not yet been established whether these kinase are important in the uPA signaling pathway, and, if so, whether the localization of these kinases to membrane rafts is important in this function. Determining whether this is so is an important goal outlined in Task 1. For this reason, we examined the molecular characteristics of a prototype raft-associated Src-family non-receptor tyrosine kinase, $p62^{Yes}$ (Yes). We found that active, tyrosine-phosphorylated Yes is present in membrane rafts (Figure 3). In Figure 3A, rafts were incubated with ^{32}P-ATP , and phosphorylated proteins were then detected by SDS-PAGE and autoradiography. Yes and a number of cellular substrates become tyrosine phosphorylated, as demonstrated by the *in vitro* kinase assay shown in Figure 3. Rafts were incubated with unlabelled ATP either on ice, as a control (Panel B, left lane) or at 37° to allow phosphorylation to occur. Proteins were then analyzed by SDS-PAGE and Western blotting, probing with anti-phosphotyrosine antibodies. Figure 3B reveals a number of substrates after the reaction. Only Yes itself is tyrosine phosphorylated before the reaction, as Yes is constitutively phosphorylated on an inhibitory site. Upon activation, it autophosphorylates a separate, activating site. To demonstrate that kinase activity was actually due to Yes, Yes was

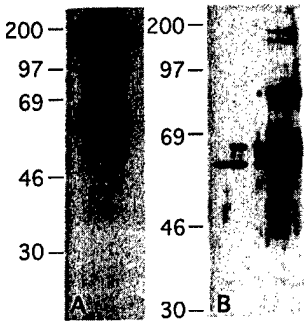


Fig. 3. Yes is active in rafts. A.; Isolated rafts were subject to an in vitro kinase assay including ^{32}P-ATP. Labeled proteins are shown in this autoradiogram. The ca. 60 kDa protein is Yes (not shown). B; Isolated rafts were incubated on ice (1st lane) or at 37° (2nd lane) with cold ATP. Tyrosine phosphorylated proteins are shown in this Western blot.

immunoprecipitated from solubilized rafts, and then subjected to the *in vitro* kinase assay. Immunoprecipitated Yes phosphorylated a number of cellular substrates (not shown). We conclude that Yes is present and active in rafts. We also note that these experiments demonstrate that Yes phosphorylates a number of substrates, many of which are likely to be important in raft-dependent uPAR signaling.

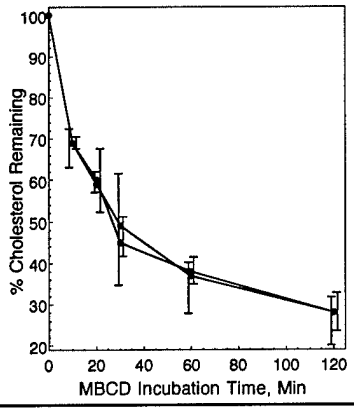


Figure 4. Cholesterol removal by MBCD. Two cancer cell lines were incubated with MBCD for the indicated times. Remaining cholesterol was measured by HP-TLC.

Task 2. Disrupt cholesterol and sphingolipid-rich rafts. This was a key goal of our proposal, and the one on which we made the most substantial progress.

MBCD rapidly and efficiently reduces cholesterol levels. As described above, one of the most effective ways of depleting cells of cholesterol and thus disrupting rafts is to treat them with MBCD. We have performed a careful analysis of the ability of MBCD to remove cholesterol from cultured cells (Figure 4). Breast cancer cells were plated in normal media and allowed to adhere to the dish. Media was then removed, and then replaced with media lacking serum or other sources of cholesterol, but containing MBCD. Preliminary experiments showed that 10 mM MBCD gave optimal effects (not shown). We also found that cells remained healthy and adherent for at least 2 hours. After 2 hours in MBCD, cells began to round up and detach from the dish. For this reason, all of our further experiments were performed at times shorter than 2 hours. At various times after addition of MBCD, individual plates of cells were harvested, after washing several times to remove cholesterol-MBCD complexes. We then extracted lipids from the cells and analyzed them, using a quantitative high-performance thin layer chromatography (HP-TLC) assay that we have used successfully in the past (Brown, 1992; Melkonian *et al.*, 1995). In this assay, lipids are separated by running in two solvent systems sequentially. The first system separates the more polar lipids, while the second separates the less polar lipids (including cholesterol). A standard curve, consisting of purified lipids in known amounts, is run separately on the same plate. The plate is then sprayed with cupric acetate in a phosphoric acid-containing solution, and heated to char the lipids. Under these conditions, the amount of charring is directly proportional to the amount of lipid. Charring can be measured using a densitometer. It is thus possible to quantitate all the lipids in the cell, using a rapid and sensitive technique. We extracted total cellular lipids from cells treated for various times with MBCD and analyzed them by this method. Importantly, lipids other than cholesterol served as internal controls. We were able to verify that levels of all lipids other than cholesterol remained constant over the course of the assay (not shown). Thus, changes in cholesterol levels were significant, and did not simply reflect cell loss. Results (Figure 4) clearly show that MBCD rapidly and efficiently selectively removes cholesterol.

MBCD treatment reduces raft-association of the GPI-anchored protein, PLAP. Another goal in Task 2 was to measure the partitioning of several molecules between rafts and Triton-soluble fractions after rafts disruption by several means. We now report the effect of raft disruption by MBCD treatment on this association, using the GPI-anchored protein PLAP as a model. Cells expressing PLAP were treated with 10 mM MBCD for varying lengths of time, up to 60 min, as indicated in Figure 5. We then extracted the cells with Triton X-100, adjusted the lysates to a high concentration of sucrose, placed them in ultracentrifuge tubes, overlaid them with solutions containing lower concentrations of sucrose to form a step gradient, and centrifuged to equilibrium. This procedure causes detergent-resistant membranes (DRMs) to float to a low-density position

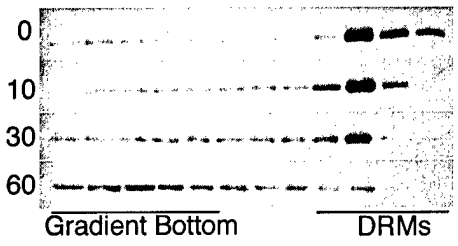


Figure 5. MBCD reduces raft-association of the GPI-anchored protein PLAP. Cancer cells expressing PLAP were incubated for various times (in min) with MBCD before preparation of DRMs by flotation on sucrose gradients. Non-DRM proteins remain at the bottom of the gradient.

high in the gradient (Brown, 1992). Gradients were fractionated, and samples of each fraction were subjected to SDS-PAGE and Western blotting analysis, probing for PLAP (Figure 5). In control cells, PLAP is highly concentrated near the top of the gradient, in the DRM-containing fractions. As cholesterol is progressively removed by incubation with MBCD for longer times, it is clear that more PLAP remains in the bottom fractions of the gradient, corresponding to the fully-solubilized non-raft (non-DRM) fractions. By 60 min, at least 50% of the protein is in these non-raft fractions. This results shows that a GPI-anchored protein is displaced from rafts upon cholesterol depletion.

Time in CD, min	% of Initial [^{3}H]Chol Remaining	% of [^{3}H]Chol in DRMs
0	100	58 ± 3
10	63 ± 15	44 ± 7
30	46 ± 11	40 ± 6
60	32 ± 2	31 ± 6

Table I. Cells prelabeled with [^{3}H]cholesterol were treated with MBCD for the indicated times and then extracted with Triton X-100. Lysates were subjected to sucrose gradient ultracentrifugation, fractionated, and radioactivity was measured.

<u>MBCD treatment reduces the raft-association of the cholesterol that remains in the cell</u>. MBCD is known to preferentially remove non-raft cholesterol from cells. However, cholesterol is expected to be in equilibrium between raft- and non-raft domains in the cell membrane. Thus, removing cholesterol – even from non-raft domains- is expected to quickly deplete raft cholesterol pools as well, leading to at least partial raft disruption. To test this idea, we developed a method for analyzing the fraction of the cholesterol still in the cell at various times after MBCD addition that was in rafts. To do this, we took advantage of the fact that cells can be labeled with ^{3}H cholesterol. As this cholesterol cannot be degraded or metabolized by cells (except to cholesteryl ester, expected to occur for only a small fraction of total cholesterol), the ^{3}H cholesterol that is added will equilibrate among all the cholesterol-containing membranes in the cell. It will thus act as a good marker for total cellular cholesterol. Cells were labeled to steady-state with ^{3}H cholesterol in this manner. They were then treated with MBCD for varying times, extracted with Triton X-100, and subjected to sucrose density gradient centrifugation to equilibrium as described above to separate raft and non-raft fractions. Gradients were fractionated, and radioactivity was measured. Data are presented in Table I. By summing all the counts in the gradient, and comparing with the untreated control, we were able to determine the fraction of total ^{3}H-cholesterol that remained in the cells at each time. (This agreed well with the amounts determined by HP-TLC.) Furthermore, by summing counts in raft and non-raft fractions respectively, we were able to determine the fraction of the total ^{3}H-cholesterol remaining in the cells at any time that was present in rafts. This clearly showed that the fraction of cholesterol present in rafts decreased with increasing time in MBCD. Together, results shown in Figure 5 and Table I show that treating cells with MBCD causes a progressive release of both a GPI-anchored proteins and cholesterol from rafts.

<u>Alternate raft-disruption methods</u>. One concern of using MBCD is that cholesterol depletion may have pleiotropic effects on cells. Cholesterol may constitute up to 50% of the total lipid in the plasma membrane on a mole basis. Although cells remain viable after MBCD treatment, it is possible that cholesterol removal has subtler and more transient effects on a number of cellular

processes. In addition to disrupting rafts, then, cholesterol removal with MBCD could easily affect other functions that relied simply on cholesterol mass in the membrane, or on specific interactions of cholesterol itself with individual membrane proteins. This is a concern for two reasons. First, our goal (outlined in the Statement of Work) is to determine the importance of rafts themselves in signaling and metastasis in breast cancer cells. If cholesterol depletion affects signaling through some other means, rather than by disrupting rafts, then further treatments and therapies based on raft disruption might not be relevant. Second, although cholesterol-depleted cells are viable in the short term, it is well-known that cholesterol is essential for long-term health on the cellular and organismal level. Thus, simply extracting cholesterol is unlikely to be a useful means of therapy for breast cancer, even if rafts are disrupted and uPAR signaling is blunted. This is because cholesterol depletion is likely to be harmful to cells for reasons that have nothing to do with rafts. For this reason, we have explored an alternate, though related, method of disrupting rafts. A number of compounds have been identified that are structurally related to cholesterol. Some of these are natural products, and fill the sterol requirement of organisms other than mammals. For instance, the commonly-studied yeast Saccharomyces cerevisiae contains ergosterol instead of cholesterol, while plants contain sitosterol. In each eukaryotic organism, the relevant sterol makes up a similarly high fraction of the total plasma membrane lipid, and performs a similar function. In addition, a number of artificial sterol analogs, similar in overall structure to cholesterol, have also been developed. Work of the London group has shown that some of these can replace cholesterol in enhancing formation of rafts in model membranes (Xu and London, 2000; Xu *et al.*, 2001). Importantly, other sterol analogs have no effect, and some even disrupt rafts formation. That is, lipid mixtures have less tendency to form rafts than they would with no sterol at all. We found that many of these compounds can bind to MBCD like cholesterol. Furthermore, MBCD-sterol complexes can deliver several of these to the plasma membranes of cells form which cholesterol has previously been depleted using MBCD. Thus, it is possible to replace cholesterol with similar amounts of another natural or artificial sterol, that either has the ability to form rafts, or has the ability to disrupt rafts. This is likely to overcome many of the pleiotropic effects of cholesterol depletion, because cells repleted with a sterol analog will have the same sterol mass as untreated cells. By choosing the appropriate analog, it should be possible to replace other essential functions of cholesterol (for instance, interactions with specific proteins (Murata *et al.*, 1995; Thiele *et al.*, 2000)) while still achieving efficient raft disruption. This could effectively block uPAR signaling and other raft-dependent functions, while preserving other essential functions of sterols in membranes.

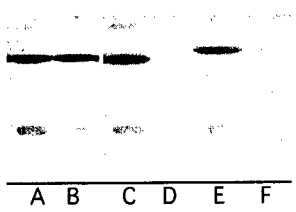


Fig. 6. TLC analysis of liposomes containing: DOPC + DPPC + cholesterol (A), DRMs prepared from those liposomes (B), DOPC + DPPC + coprostanol (C), DRMs from those liposomes (D), DOPC + DPPC + androstanol (E) or DRMs from those liposomes (F).

For this reason, we examined a panel of sterol analogs. For initial characterization, we examined the raft-forming behavior of these sterols in model membranes. To assay raft association, and the effect of the sterols on rafts, we used the detergent-insolubility assay that we have developed previously (Brown and Rose, 1992; Melkonian *et al.*, 1995; Brown and London, 1998b; Ostermeyer *et al.*, 1999; Brown and London, 2000; Shogomori and Brown, 2003). Androstanol and coprostanol were found to disrupt rafts, and were not included in detergent-resistant membranes (DRMs) prepared from model membranes (Figure 6). By contrast, like cholesterol (Figure 6), dihydrocholesterol and ergosterol (not shown) were enriched in the DRMs, showing that they associate tightly with rafts. Liposomes contained the raft-forming phospholipid DPPC (which does not char, so is not detected on the TLC plate, but was enriched in the DRMs), the non-raft-forming

phospholipid DOPC (present in the starting liposomes, lanes A, C, E, and visible as the lower band, but extracted by Triton X-100 and not visible in the DRMs, lanes B, D, and F) and sterols, either cholesterol (lanes A and B), coprostanol (lanes C and D) or androstanol (lanes E and F). Sterols are visible as the dark upper band. (The faint uppermost band is an unidentified contaminant). Note that although cholesterol is enriched in DRMs prepared from the liposomes (lane B), both coprostanol and androstanol are completely solubilized, and not detectable in the DRMs (lanes D and F), demonstrating their lack of raft association in vitro.

Sphingomyelinase treatment efficiently reduces sphingomyelin levels. We have shown that rafts require both cholesterol and sphingolipids (Ahmed *et al.*, 1997; Schroeder *et al.*, 1998). Thus, an alternate method of disrupting rafts is to deplete cells of sphingolipids. The enzyme sphingomyelinase rapidly and efficiently cleaves the head group from sphingomyelin, destroying it. Cells are rapidly depleted of sphingomyelin, allowing examination of acute effects of this treatment. There are two major classes of sphingolipids in mammalian cells: sphingomyelin and glycosphingolipids. Sphingomyelinase, as mentioned above, rapidly destroys sphingomyelin. However, there are no comparable methods for destroying glycosphingolipids. Thus, glycosphingolipids remain after sphingomyelinase treatment, complicating interpretation of the results. Fortunately, we have obtained a very useful line of cancer cells that cannot make glycosphingolipids. These were developed by Hirabayashi and colleagues (Ichikawa *et al.*, 1994). Although these are melanoma cells and not breast cancer cells, we believe that principles derived from melanoma cells will apply to breast cancer cells as well. For this reason, we have treated these cells with sphingomyelinase. Cells were treated for 30 minutes with 5 units/ml sphingomyelinase (or were treated with vehicle alone as a control). This short time assured that accumulation of signaling compounds generated from sphingomyelin breakdown didn't affect the results. We then extracted lipids and subjected them to quantitative HP-TLC as described above. Figure 7 shows the amount of sphingomyelin in treated and control cells, visualized by charring. Quantitation of the results by comparison to standards revealed that 80% of the total cellular sphingomyelin was degraded by this method.

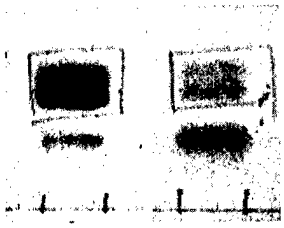
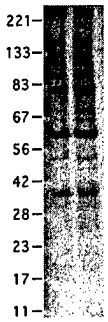


Figure 7. Sphingomyelinase removes most sphingomyelin. Cells were treated (right) or not (left) with sphingomyelinase. Lipids were extracted and analyzed by HP-TLC (origin marked with a line), visualized by charring. The sphingomyelin doublet is boxed.

Sphingolipid depletion does not fully disrupt rafts. As the cells described above lack glycosphingolipids, sphingomyelinase treatment reduces total sphingolipid levels by 80%. This is about the same as the level of cholesterol depletion by MBCD. As described above, this level of cholesterol disruption significantly affects the properties of rafts. However, we obtained an unexpected result when we examined sphingolipid depletion. We first used our standard detergent insolubility assay for raft recovery. Lysates were then adjusted to a high density of sucrose and placed in an ultracentrifuge tube. A sucrose gradient was layered over the lysate, and was spun to equilibrium. Detergent-insoluble rafts were recovered from the interface between medium-density and low-density steps in the gradient (Melkonian *et al.*, 1995). Surprisingly, we visually observed good recovery of detergent-resistant membrane (DRMs) rafts, even after sphingomyelinase treatment (not shown). As this is a non-quantitative measure of DRM yield, we also examined the protein profile of these DRMs, as a way of determining both quantitatively whether protein yield in DRMs was reduced by sphingomyelinase treatment, and whether different proteins were extracted to different extents before and after treatment. Cells containing proteins labeled to steady-state with ^{35}S-methionine, with (Figure 8, right) or without (Figure 8, left) sphingomyelinase treatment, were



lysed in cold Triton X-100, as we have described earlier (Brown and London, 1998b). Results (shown in Figure 8) revealed that very similar DRM protein profiles were obtained in each case. Thus, in contrast to cholesterol depletion, cleavage of sphingomyelin in glycosphingolipid-negative cells had little detectable effect on the ability of proteins to associate with DRMs. We speculate that this may result from the ability of ceramide, which is generated from sphingomyelin after cleavage, to support raft formation.

Figure 8. Effect of sphingomyelinase treatment on DRM protein profile. ^{35}S-methionine-labeled cells were treated (right lane) or not (left lane) with sphingomyelinase before DRMs were prepared and DRM proteins analyzed by SDS-PAGE and autoradiography.

Cell-surface distribution of raft components. Examining cell-surface distribution of raft markers is one of the most powerful and useful ways of determining the integrity of rafts in cells (Harder *et al.*, 1998; Janes *et al.*, 1999; Viola *et al.*, 1999). Most raft markers appear to be relatively uniformly distributed on the surface of resting cells. This is because rafts in these cells are small and highly dispersed. Only during signaling, when raft proteins become clustered together (for instance, through binding to ligand and subsequent dimerization and oligomerization) do rafts become apparent (Harder, 2001). For reasons that are still not completely clear, during signaling events, the small, fairly unstable rafts present on resting cells aggregate together into much larger, stabler raft structures. These are easily visible by light microscopy, using fluorescently-tagged antibodies or other reagents as probes. Two classes of raft markers are the most commonly used for these studies. The first are GPI-anchored proteins. Antibodies directed against these proteins are added to the surface of live, unfixed cells and allowed to bind to their targets. Excess unbound antibodies are then washed away. Next, secondary antibodies (antibodies raised in a different species, that recognize the first or primary antibodies, which themselves recognize the antigen) are added and allowed to bind to the primary antibodies. Importantly, because the secondary antibodies are polyclonal, individual antibody molecules can recognize a variety of different sites on the primary antibody molecule. Furthermore, as each secondary antibody is bivalent, it can simultaneously bind two identical sites, on two different primary antibody molecules. The net effect of these two factors is that secondary antibodies induce clustering of primary antibodies, and – by extension- of the receptor molecule on the surface of the cell that served as the antigen for the primary antibody. This antibody-induced clustering is a useful mimic of physiologically relevant receptor clustering that occurs following ligand binding in cells, and is often used as a convenient model of this clustering. We have established this read-out system in our lab for PLAP, as shown in Figure 9. Panel A shows that in untreated cultured breast cancer cells expressing PLAP, the protein has a uniform distribution on resting cells. After antibody-induced clustering, however, the protein has a dramatically different distribution, as shown in Panel B. It is now observed in large, discrete, ragged clustered patches. These are much larger than would be expected for simple clustering of the protein alone. Instead, the fact that the protein binds rafts causes the small rafts to coalesce together and become stabilized, leading to formation of much larger clustered structures than would be seen otherwise.

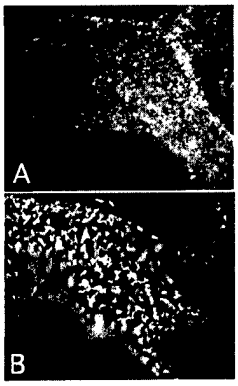


Fig. 9. Effect of antibody-mediated clustering on PLAP distribution. (A), as in Fig. 1. (B), before fixation, rabbit anti-PLAP antibodies and then fluoresceinated goat anti-rabbit antibodies were added to MCF-7 cells transfected with PLAP.

The second tool for clustering rafts takes advantage of the fact that gangliosides (acidic glycosphingolipids), which are highly concentrated in rafts, can be bound by a number of bacterial

toxins. Many of these toxins are targeted to rafts by ganglioside binding, and this raft targeting is essential for cell entry and intoxication (Wolf *et al.*, 1998). A useful toxin is cholera toxin. This toxin consists of 2 subunits, A and B. The pentameric B subunit binds 5 molecules of the ganglioside GM1 or certain other related gangliosides, causing them to cluster together. Subsequent to this binding, entry of the A subunit into cells leads to toxic effects. Experimentally, the isolated B subunit can be used to cluster gangliosides, without danger of the toxicity that would derive from the A subunit. The cholera toxin B subunit can be labeled directly with fluorescent probes such as fluorescein for detection using fluorescence microscopy. Alternatively, using the same approach as described above for antibody-mediated clustering of membrane proteins, antibodies directed against the cholera toxin B subunit can be used to further enhance clustering (Harder *et al.*, 1998; Janes *et al.*, 1999).

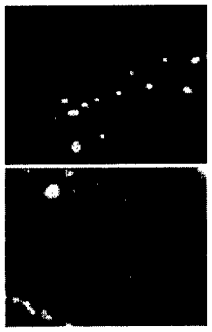


Fig. 10. Filipin disrupts cholera toxin clusters. MCF-7 cells were fixed with paraformaldehyde, and cholera toxin fluorescence was observed directly (top) or after treatment with filipin (bottom).

Distribution of clustered raft components on cells is affected by raft integrity. We used this system as a probe for the efficiency of raft disruption by the polyene antibiotic filipin. Filipin inserts into membranes and then forms complexes with cholesterol. In doing so, it prevents cholesterol from engaging in the other types of lipid interaction, including those required for raft formation. Thus, treating membranes with filipin should disrupt rafts. When individual raft components are clustered with antibodies or toxins, much smaller clusters are expected. These small clusters derive only from direct association of antibody and/or toxin molecules with their membrane targets, without the additional effect of raft clustering.

We first examined the effect of filipin treatment on GM1 clustering. Cholera toxin covalently linked to the fluorescent dye fluorescein was added to the surface of cells. Cells were maintained at a low temperature (15°), to avoid endocytosis and internalization of the toxin, as this would complicate analysis. Once bound, excess toxin was washed away. Next, antibodies directed against the toxin were added. Even at reduced temperature, the diffusion rate of the lipid in the membrane was sufficient to allow clustering. After incubating for 1 hour to allow binding and clustering, excess antibodies were washed away. Cells were fixed with paraformaldehyde, and cholera toxin fluorescence was observed as shown in Figure 10 (top). Large, fairly dispersed puncta are visible. The experiment was then repeated in the presence of 100 micrograms/ml of filipin. Under these conditions, much smaller, "fuzzier" puncta are seen (Figure 10, bottom). Similar results were obtained when rafts were disrupted with methyl-beta cyclodextrin (MBCD), which efficiently removes cholesterol from membranes (not shown). This result clearly shows that rafts are efficiently disrupted by these agents, and that the distribution of clustered GM1, visualized microscopically, is a useful tool for monitoring raft disruption.

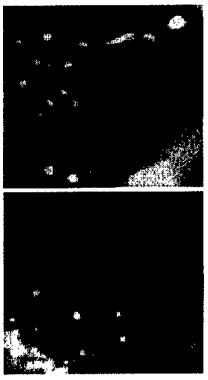


Fig. 11. Filipin disrupts PLAP clusters. MCF-7 cells expressing PLAP were incubated with anti-PLAP antibodies and fluorescent goat anti-rabbit antibodies without (top) or with (bottom) filipin treatment before fixation.

We also examined the effect of raft disruption on cell-surface distribution and clustering of the GPI-anchored protein PLAP. Rabbit polyclonal antibodies directed against PLAP were added to cells maintained at reduced temperature (15°), and cells were incubated for 1 hour to allow antibody binding. Excess antibodies were washed away, and fluorescein-conjugated secondary antibodies (goat anti-rabbit IgG) were added and incubated

for one hour. As described above, this procedure allowed crosslinking of the primary antibodies along with their bound ligands, further enhancing clustering. Because the secondary antibodies were linked to fluorescein, they also served as the detection probe.

Large, dispersed puncta were observed after clustering (Figure 11, top), as in Figure 9. These were generally larger than the GM1 clusters shown in Figure 10. This may result from the fact that PLAP, as a protein, has more epitopes and can be clustered more efficiently by polyclonal antibodies than the structurally simpler GM1. Alternatively, the affinity of PLAP for rafts may be greater than GM1. (PLAP is a dimer, which would be expected to increase its raft affinity. However, the fact that cholera toxin is pentameric may argue against this possibility). It is even possible that GM1 and PLAP can inhabit different types of raft, although this possibility would require further study outside the scope of this project.

We next repeated the experiment, treating cells with 100 micrograms/ml filipin during both antibody-binding steps. Under these conditions, much smaller, dispersed puncta were observed (Figure 11, bottom). These were very similar to the GM1 puncta after filipin treatment. We conclude that filipin efficiently disrupts rafts and prevents raft clustering. Both GM1 and the GPI-anchored protein PLAP are useful markers of this effect.

Caveolin-1: a raft protein down-regulated in cancer. We took one additional approach to the important question of raft disruption. That was to examine the role of the membrane protein caveolin-1. Caveolin-1 forms a coat surrounding the surface of 50-100 nm pits or invaginations in the plasma membrane of cells called caveolae. Caveolin-1 forms high-molecular weight oligomers that associate laterally with each other for form filaments that line the surface of caveolae. Because caveolin-1 has a high affinity for rafts, rafts become concentrated in caveolae. That is, as small, unstable rafts diffuse in the plasma membrane, they tend to remain in caveolae because of their affinity for caveolin-1. The high concentration of caveolin-1 in caveolae has the same effect as antibody (or ligand) on causing rafts to coalesce together and stabilizing them. Thus, the entire membrane bilayer in caveolae is probably in the form of a raft. A number of signaling events are concentrated in caveolae (Smart *et al.*, 1999), and they serve as signaling centers at the plasma membrane, concentrating together proteins that need to work together in signaling. Importantly, uPAR is especially highly concentrated in caveolae, reinforcing our hypothesis that association with rafts in the specialized caveolae environment is crucial for uPAR function. Caveolin-1 is often down-regulated in cancers, and caveolae – which depend on caveolin-1 for their formation - suggesting that a down-modulation of signaling (or of regulation of signaling) can enhance tumor progression. However, one particular caveolin-1 mutant (P132L) has been found in 16% of human scirrhous breast cancer lines (Hayashi *et al.*, 2001). We found that P132L lost the ability to associate with rafts, as measured by the fact that it was not enriched in detergent-resistant membranes (DRMs) prepared from cells expressing the protein. This assay is described in detail next. Cells that do not normally express caveolin-1 are transiently transfected with either wild-type caveolin or a mutant – in this case, P132L. Cells are then lysed with buffer containing the non-ionic detergent Triton X-100 on ice. Lysates are then spun at high speed in the centrifuge. The detergent-solubilized material (that does not associate with DRMs) remains in the supernatant, while DRMs (along with cytoskeleton) pellets. Aliquots of the supernatant and pellet fractions are analyzed by SDS-PAGE and Western blotting, probing with anti-caveolin antibodies. The relative amount of the protein in the two fractions shows the affinity of the protein for DRMs and rafts. As shown in Figure 12, wild-type caveolin-1 was enriched in the

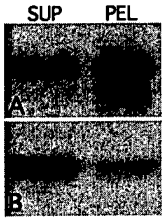


Fig. 12. DRM-association of caveolin-1 and the P132L mutant. MCF-7 cells expressing wild-type caveolin-1 (A)

pellet fraction (pel) while P132L (Panel B) was enriched in the supernatant fraction. This suggested that the prevalence of P132L in breast cancer might be related to its loss of raft affinity. Because caveolin-1, unlike other raft proteins, actively organizes and affects the structure of membrane rafts, it might be necessary for setting up the specific membrane lipid microenvironment required for uPAR signaling.

Task 3. Apply the raft-disruption methods in Task 2 to the uPAR functional assays in Task 1. The difficulty in detecting uPAR with available antibodies, described in the section of the report on Task 1, prevented us from making significant progress toward this goal. Nevertheless, the advances reported above in understanding the relationship between cell-surface distribution, raft association, and signaling of GPI-anchored proteins will greatly facilitate further studies of the specific role of raft association in uPAR signaling.

KEY RESEARCH ACCOMPLISHMENTS

As detailed in the Body, the key research accomplishments resulting from this study are the following findings:

- Raft disruption by cholesterol depletion stimulates the MAP kinase pathway.
- Yes is present and active in membrane rafts, and tyrosine phosphorylates a number of cellular proteins.
- MBCD rapidly and efficiently reduces cholesterol levels.
- MBCD treatment reduces raft-association of the GPI-anchored protein, PLAP.
- MBCD treatment reduces the raft-association of the cholesterol that remains in the cell.
- Raft disruption by MBCD treatment can be detected by fluorescence microscopy
- Sphingomyelinase treatment efficiently reduces sphingomyelin levels.
- Sphingolipid depletion does not fully disrupt rafts
- Sterol analogs such as coprostanol and androstanol can be used to disrupt rafts

REPORTABLE OUTCOMES

The principle investigator gave presentations including work described here at the following venues:

6/12/00; Cutaneous Biology Research Center, Massachusetts General Hospital, Charlestown, MA
7/27-7/29 2000; Japanese Society of Carbohydrate Research Annual meeting, Nagoya, Japan
8/6-8/11 2000; FASEB (Federation of American Societies for Experimental biology) summer conference on "Lipid-Modifications of Proteins", Copper Mountain, CO
10/23-24/00; Department of Biochemistry, University of Alberta, Edmonton, Canada
10/30/00; Biology Department, University of Vermont, Burlington, VT
2/18/01; Biophysical Society Annual meeting, Boston, MA
3/21/01; Department of Physiology, Indiana University, Indianapolis, IN
4/6/01; Graduate Seminar Program, Southern Methodist University, Dallas, TX
09/10/01; Dept. of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN
09/25/01; Biochemistry Dept., Boston University School of Medicine, Boston, MA
10/09/01; Integrated Graduate Program, Northwestern University School of Medicine, Chicago, IL
01/16/02; Oregon Health & Sciences University, Dept Physiology and Pharmacology, Portland, OR

02/04/01; Dept. Physiology & Biophysics, Robert Wood Johnson Medical School, Piscataway, NJ
02/28/02; Biochemistry Dept., Case Western Reserve University, Cleveland, OH
04/04/02; Biochemistry and Molecular Biology Dept., Michigan State University, E. Lansing, MI
4/15/02 US Army Medical Research Institute of Infectious Diseases (USAMRIID) Frederick, MD
10/9/02 Johns Hopkins University, Pharmacology Department, Baltimore, MD
1/15/03 Dartmouth University, Pharmacology Department Hanover, NH
3/24/03 Children's Hospital, Boston, MA
6/9/03 Albany Medical College, Albany, NY
9/15/03 St. Louis University, St. Louis, MO
No publications resulted from this work.

CONCLUSIONS

The medical significance of this work remains as described in the original proposal. It is clear that uPA interactions with uPAR play a key role in metastasis, the deadliest feature of breast cancer. Our findings strongly suggest that the presence of uPAR, a GPI-anchored protein, in membrane rafts affects its signaling and its ability to govern cell migration during metastasis. As methods for disrupting rafts are becoming more readily available, the importance of testing the ability of these compounds to inhibit uPAR signaling has never been greater.

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